# A high-conductance mode of a poly-3-hydroxybutyrate/calcium/polyphosphate channel isolated from competent *Escherichia coli* cells

Evgeny Pavlov*, Chelsey Grimbly, Catherine T.M. Diao, Robert J. French

*Hotchkiss Brain Institute, Department of Physiology and Biophysics, Faculty of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alta., Canada T2N 4N1*

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**Abstract Reconstitution into planar lipid bilayers of a poly-3-hydroxybutyrate/calcium/polyphosphate (PHB/$Ca^{2+}$/polyP) complex from *Escherichia coli* membranes yields cationic-selective, 100 pS channels (Das, S., Lengweiler, U.D., Seebach, D. and Reusch, R.N. (1997) Proof for a non-proteinaceous calcium-selective channel in *Escherichia coli* by total synthesis from (*R*)-3-hydroxybutanoic acid and inorganic polyphosphate. Proc. Natl. Acad. Sci. USA 94, 9075–9079). Here, we report that this complex can also form larger, weakly selective pores, with a maximal conductance ranging from 250 pS to 1 nS in different experiments (symmetric 150 mM KCl). Single channels were inhibited by lanthanum ($IC_{50}$ = 42 ± 4 μM, means ± S.E.M.) with an unusually high Hill coefficient (8.4 ± 1.2). Transition to low-conductance states (<250 pS) was favored by increased membrane polarization ($|V| \geqslant 50$ mV). High conductance states (>250 pS) may reflect conformations important for genetic transformability, or "competence", of the bacterial cells, which requires the presence of the PHB/$Ca^{2+}$/polyP complex in the membrane.**



*Keywords:* Polyphosphate; Reconstitution; Planar lipid bilayers; *Escherichia coli*

## 1. Introduction

The poly-3-pydroxybutyrate/calcium/polyphosphate (PHB/$Ca^{2+}$/polyP) complex was first discovered in the membranes of competent *E. coli* cells [1]. This complex is formed of two polymers, PHB and polyP, presumed to be linked together via $Ca^{2+}$ ions by ionic bonds. Reusch and collaborators [2] have modeled the channel structure as a pair of concentric cylinders, with the PHB molecule forming an outer sheath, which spans the membrane and offers a hydrophobic surface to the surrounding lipid, and polyP located inside, linked to the inner hydrophilic face of the PHB by $Ca^{2+}$. Possible conformations for the PHB and polyP polymer chains have been critically discussed [2,3], but structural details remain to be confirmed. In experiments with PHB/$Ca^{2+}$/polyP derived from *E. coli*, and with synthetic polymers, the complex can form channels when reconstituted into lipid bilayers. Channels formed by the PHB/$Ca^{2+}$/polyP complex were found to be cationic selective with a preference for calcium and ~100 pS conductance [3].

Recently, we found that a similar PHB/$Ca^{2+}$/polyP complex, isolated from rat liver mitochondria, can form large (mean maximal conductance 500 pS in 150 mM KCl), voltage-dependent, weakly selective pores with multiple sub-conductances, including values of ~100 pS. Behavior of this channel in the high conductance range, in many respects, mimicked the behavior of mitochondrial permeability transition pores seen in patch-clamp experiments of native mitoplasts, suggesting an important physiological role [4].

The similar chemical composition of mitochondrial and bacterial PHB/$Ca^{2+}$/polyP complexes suggests that the complex from *E. coli* might also form large conductance channels. Here, we report that the PHB/$Ca^{2+}$/polyP from *E. coli* can indeed form large conductance channels, at low voltage, when reconstituted into lipid bilayers. Although behavior of the bacterial channels was similar to those from mitochondria, significant quantitative differences were identified. These included, for the bacterial channel, stronger selectivity for cations, smaller conductance and a larger voltage for transition to the lower conductance range. We propose that these differences may arise, in part, from the difference in size of polyP molecules contributing to the complexes in bacterial and mitochondrial channels.

In addition to new insight into the role played by polyP in defining the properties of PHB/$Ca^{2+}$/polyP channel, we suggest that newly recognized high conductance state(s) might play an important physiological role. It has been shown that the presence of the PHB/$Ca^{2+}$/polyP complex in the membrane is essential for bacterial competence [5]. Native bacterial membranes contain channels which are permeable to DNA [6] and show conductances comparable to those seen in our experiments. This suggests that such channels might directly participate in the process of DNA import during bacterial transformation.

## 2. Materials and methods

### 2.1. Reconstitution of PHB/$Ca^{2+}$/polyP complex and electrophysiological studies

For bilayer recordings, PHB/$Ca^{2+}$/polyP complex was isolated from competent *E. coli* DH 5α cells using chloroform extraction as previously described [3]. Bilayers were formed across an aperture of

*Corresponding author. Fax: +1 403 283 8731.
*E-mail address:* epavlov@ucalgary.ca (E. Pavlov).

*Abbreviations:* $E_K$, equilibrium potential for $K^+$; $E_{rev}$, reversal potential; PHB, poly-3-hydroxybutyrate; polyP, polyphosphate

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100–200 μm diameter in the wall of a Teflon cup (Warner Instruments) by painting with a mixture containing chloroform extract of competent *E. coli* cells and POPE (Avanti) diluted in decane to the concentration of 10 mg lipid/ml. The sign of the voltage is that of the opposite (trans) chamber from which the lipid/chloroform mixture was painted, and cannot be rigorously associated with a particular channel orientation. Experiments were done using silver-chloride electrodes without salt bridges. Net junction potentials were balanced to zero before bilayer formation and tested again after membrane disruption at the end of each experiment. In all experiments shown, junction potential drift was less than 2 mV and was not considered significant. Uncompensated junction potentials for 150–50 mM gradient of KCl were estimated to be 0.5 mV using JPCALC [7], supplied with the pClamp software. All recordings were done at room temperature. In experiments to test for possible state-dependent reversal potential shifts, the concentration gradient was created and the junction potential offset was compensated before membrane painting. Solutions were not changed during an experiment, so absolute values of half-cell potentials at the Ag/AgCl surfaces had no effect on measurements of shifts in reversal potential. Recording solutions contained either 200 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM Tris–HEPES; or 150 mM KCl, 5 mM Tris–HEPES (in some experiments, 1 or 5 mM of $MgCl_2$ was added to this solution; this did not affect observed conductances.). All solutions were adjusted to pH 7.4. Data were collected with Axopatch-1B amplifier, filtered at 100 Hz (−3 dB, low pass, 4-pole Bessel filter) and digitally recorded to PC, using pClamp 9.0 software (Axon Instruments, Union City, CA). Most analysis was performed with the same software. Summary data are expressed as means ± S.E.M. (no. of determinations).

### 2.2. Polyphosphate extraction and chain length determination

For gel analysis, polyphosphate was extracted from membrane fractions from either competent or non-competent *E. coli*. Bacteria were resuspended in 8 ml of either transformation buffer (for competent cells) or Difco™ SOB medium (for non-competent cells). The transformation buffer contained 100 mM KCl, 10 mM $CaCl_2$, 45 mM $MnCl_2$ and 10 mM MES-KOH pH 6.3. Two milli litre of the suspension was added to a beater tube along with 0.75 g of microbeads with a diameter of 0.1 mm (Biospec Products). The mixture was placed in a bead beater (Biospec Products) for 1 min at the highest mixing speed and then on ice for 1 min. This procedure, carried out in a cold room at 4 °C, was repeated 10 times. The suspension was then spun at $13000 \times g$ for 30 min and the supernatant was discarded. A mitochondrial membrane fraction was isolated from purified rat liver mitochondria using osmotic shock as previously described [8]. Poly-P was extracted from the membrane fractions from *E. coli* or mitochondria into the aqueous phase of chloroform–methanol–water (8:4:1) mixture.

Polyphosphates were visualized by toluidine blue staining of 15% PAGE gels (acrylamide:bis, 19:1) run in 90 mM Tris–borate, pH 8.3 with 2.7 mM EDTA. The approximate length of the polyphosphate was estimated by comparison with dye standards. For the 15% gel, the position of xylene cyanol (XC) corresponds to a polyphosphate molecule of 72 phosphate groups, and the position of bromophenol blue (BPB) to 35 [9].

## 3. Results

Calcium is a key structural element of PHB/$Ca^{2+}$/polyP complex. This ion participates in complex formation by linking the two polymers through ionic bonds with phosphoryl oxygen atoms of the polyP and ion-dipole bonds with the ester carbonyl oxygens of the PHB [1]. For this reason, channel properties of the complex should depend strongly on the concentration of calcium ions (or other divalent or polyvalent cations) in the recording media. Most of the studies of bacterial and synthetic PHB/$Ca^{2+}$/polyP channel properties have been done using high concentrations of calcium in the recording solutions (for example see [3,10]). On the other hand, recordings of native bacterial channels have been typically performed in KCl recording media with low calcium present [6]. In this study, we examined channel properties in both types of solutions: high calcium, containing 200 mM $CaCl_2$ and 5 mM $MgCl_2$, and high potassium, containing 150 mM KCl, in some cases with added $MgCl_2$ (1–5 mM). Records were made over a range of voltage which, for mitochondrial channels, revealed several conducting states and kinetic modes, in order to see if channels in the bacterial membrane extract showed similar behavior.

Fig. 1 presents typical single channel data from the reconstituted PHB/$Ca^{2+}$/polyP complex in the presence of 200 mM $CaCl_2$. All traces shown in Fig. 1A were collected from the same experiment. Fig. 1A shows recordings of single channel activity at different voltages. At lower voltages, channel conductance was about 700 pS and the channel stayed near its maximal conductance state. At larger voltages, the channel entered lower conductance sub-states (Fig. 1A and B). Application of a steady ramp command voltage showed the voltage dependence of the conductance to be approximately symmetric (Fig. 1C). The channel tended to switch to lower conductance states at larger absolute voltages of either polarity, as we have reported for mitochondrial PHB/$Ca^{2+}$/polyP channels [4]. At ±100 mV, we observed reversible transitions into and out of lower conductance substates of about 100 and 200 pS (Fig. 1D, two upper traces). The 100 pS sub-state demonstrates kinetics typical of the PHB/$Ca^{2+}$/polyP channel reported earlier for high calcium solutions [10]. As illustrated in Fig. 1D, 100, 200 and 500 pS states were interconvertible, and were never observed simultaneously, suggesting that they are all different conductance states of a single channel rather than reflecting presence of different ion channels. Fig. 1D (lower trace) shows another example of kinetic behavior of the 100 pS sub-state from the same experiment. Two kinetic modes are seen, with fast or slow gating, as previously reported to be typical for PHB/$Ca^{2+}$/polyP channels (see Fig. 2 of Das and Reusch [10]). Overall, we conclude that the channel we observed demonstrated behavior like that of the PHB/$Ca^{2+}$/polyP complex.

We also investigated single channel properties of the bacterial PHB/$Ca^{2+}$/polyP complex recorded in symmetric 150 mM KCl. Representative traces and corresponding all-points histograms are shown in Fig. 2A and B, respectively. At lower voltages, as for high [$Ca^{2+}$], larger conductance states were observed. The high conductance states demonstrated selectivity for cations over anions, but the reversal potential implied significant anion permeability ($E_{rev}$ = 22 mV, in 150/50 KCl). The reversal potential did not change with conductance (Fig. 2C) and was reproducible within 1 mV (five experiments). Maximal conductance varied from 200 pS to 1 nS in 39 different experiments, and a Gaussian fit of the histogram in Fig. 2D yielded an average maximal conductance of 360 ± 170 pS. As in high calcium, channels tended to switch to lower conductance states as voltage increased, with transition kinetics characteristic of PHB/$Ca^{2+}$/polyP channels (Figs. 2A and B).

Under all ionic conditions tested, the channels demonstrated voltage dependence. This was similar to that of PHB/$Ca^{2+}$/polyP channels from mitochondria, but for *E. coli* channels, higher voltages were required to observe transitions to the low conductance states (~50 mV for *E. coli* vs. ~30 mV for mitochondria, see Table 1).

The channel was inhibited by lanthanum with apparent $IC_{50}$ = 42 ± μM (Fig. 3A and B). This inhibition was reversed

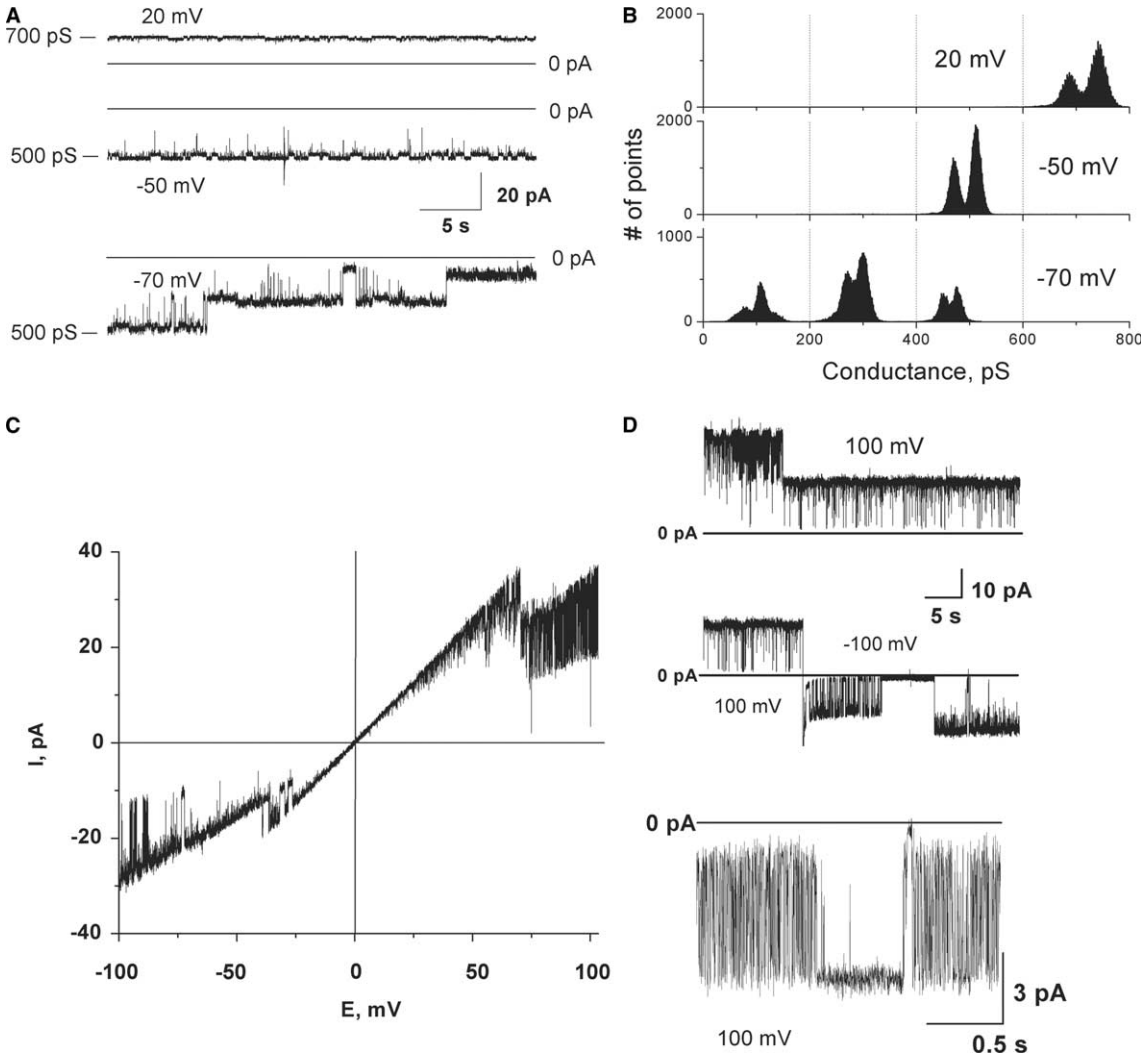


Fig. 1. Representative single channel data from the PHB/$Ca^{2+}$/polyP complex reconstituted into a lipid bilayer with recording solutions containing 200 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM Tris–HEPES, pH 7.4 (A) Single channel records showing voltage-dependent appearance of different conductance states. Bars to the left of the traces show the conductance values. (B) All-points histograms of the channel conductance, estimated from Ohm's law at different voltages, from the same experiment as in part A. Each histogram presents data from at least 25 s of record. (C) Single-channel current elicited by ramping the voltage from −100 to 100 mV. Note the higher conductance between ~ − 30 and +70 mV. (D) Channel transitions to the lower conductance sub-states at +100 and −100 mV (upper and middle traces). Lower conductance state (110 pS) with two gating modes (lowest trace), as previously reported for bacterial PHB/$Ca^{2+}$/polyP channels [10].

by either washout (data not shown) or by chelation of lanthanum by addition of EDTA (Fig. 3C). The Hill coefficient for block was 8.4 ± 1.2 ($n$ = 6) indicating high cooperativity. Both this, and the progressive, rather than stepwise, decrease in single channel current following lanthanum addition are consistent with the involvement of a large number of inhibitory sites for each channel.

Fig. 4 shows a toluidine blue gel of polyP isolated from *E. coli* and from mitochondria. The migration speed of polyP molecule depends the polymer length (see Section 2). Our data show that mitochondrial polyP is significantly shorter than polyP from *E. coli*. The band corresponding to polyP of *E. coli* origin is significantly higher than dye marker corresponding to a polyP 72-mer, giving an estimated length of polyP ~100–150, whereas the polyP from mitochondria lies between 35 and 72 dye markers.

## 4. Discussion

Recently, we reported that the PHB/$Ca^{2+}$/polyP complex isolated from mitochondria can form, in addition to low-conductance cation-selective channels, large-conductance, weakly selective pores [4]. The overall properties of the mitochondrial channel are very similar to those of the calcium-induced permeability transition pore of the intact mitochondrial membranes seen in patch clamp experiments (reviewed in [11])., They exhibit large conductance (300–700 pS in 150 mM KCl) and voltage-dependence. In the present work, we found that a PHB/$Ca^{2+}$/polyP complex from *E. coli* also forms large conductance channels. Although the general behavior of the *E. coli* channel is very similar to the mitochondrial one, significant differences in selectivity, size and voltage-dependence were detected (Table 1).

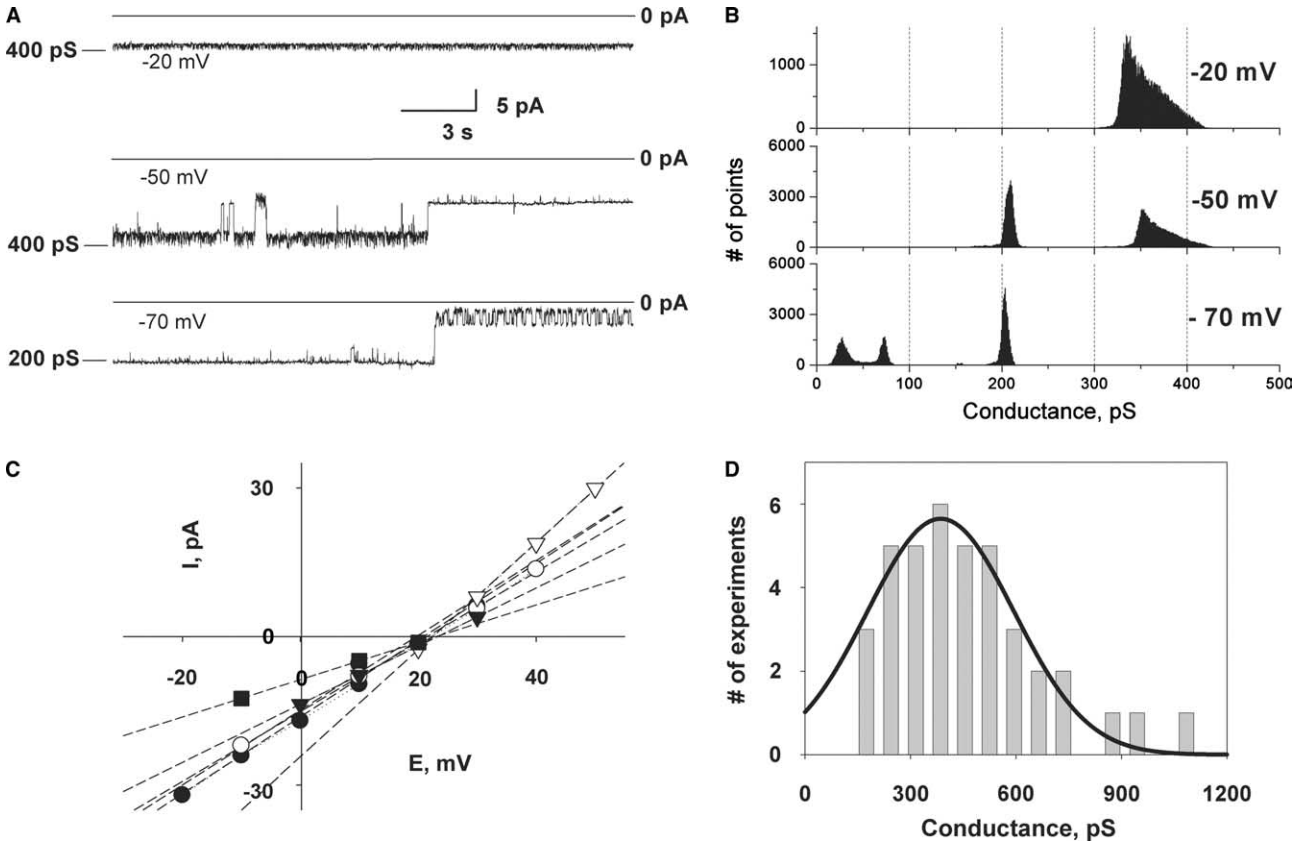


Fig. 2. Single channel characteristics of PHB/$Ca^{2+}$/polyP complex reconstituted into lipid bilayers; recording solutions contained 150 mM KCl, 5 mM Tris–HEPES, pH 7.4. (A) Transitions from the fully open state to the lower conductance sub-states were observed at −50 and −70 mV. Bars to the left of the traces indicate the conductance values. (B) All-points histograms of the channel conductance, estimated from Ohm's law, at different voltages. The asymmetry of the high conductance peak results from the filtering of the record, combined with the fact that at −20 and −50 mV, attainment of the true maximal conductance is rare, with the mode of the broad peak being ~50 pS less than the maximal value attained in brief, discrete events. This asymmetric distribution is analogous to those used for $\beta$-distribution analysis of fluctuation kinetics [16]. Each histogram presents data from at least 25 s of record. (C) *I–V* relations for the maximal conductance states from five different experiments with a KCl gradient (50:150 mM, $E_{rev} = 22 \pm 1$ mV, $E_K \sim 28$ mV). (D) Maximal conductance distribution histogram from 39 separate experiments; mean value, from Gaussian fit (solid line), is 390 ± 170 pS. The values plotted represent data from the high conductance peaks of individual experiments as illustrated for −20 and −50 mV in part (B), and include data from experiments with 0, 1 or 5 mM $MgCl_2$ (see Section 2).

Table 1
Comparison of the channel properties of PHB/$Ca^{2+}$/polyP complexes from mitochondria and *E. coli*

| | Mitochondria | *E. coli* |
|---|---|---|
| Maximal conductance, pS (150 mM KCl) | 540 ± 120 | 390 ± 170 |
| Selectivity of maximal conductance state | Variable | Cation-selective ($P_K/P_{Cl} = 10$) |
| Voltage range for appearance of the low-conductance state[a] | $\|V\| \geqslant 30$ mV | $\|V\| \geqslant 50$ mV |
| Lanthanum block | Only for sub-states | Yes |
| Apparent polyP size (# of monomeric units, from gel mobility) | 60–70 | 120–150 |

[a]The minimum voltage for appearance of the low-conductance state was determined by visual inspection of records for at least 20 s following a step to the test voltage (>20 experiments for each channel type).

An existing working model of PHB/$Ca^{2+}$/polyP complex portrays PHB polymer forming a membrane-spanning cylindrical structure, filled with polyP, with the two polymers linked together electrostatically by calcium [1]. PolyP is a flexible polymer which could have multiple conformational states inside the complex. Some of these conformations might underly the high conductance states which we observed. Experiments with synthetic components showed that PHB channels, which lack polyP, also lack selectivity and voltage-dependence [12]. In our study, the different channel properties between mitochondrial and bacterial PHB/$Ca^{2+}$/polyP complex were associated with a difference in apparent polyP length. The longer length of the polyP molecule in *E. coli* channels might be responsible for their stronger cationic selectivity and smaller average conductance. Although our data suggest a difference in polymer length, it is important to note that, as in the case of protein gels, sizes inferred from gel mobility standards do not necessarily reflect absolute polymeric lengths or molecular weights. Another study has reported polyP, which confers competence on *E. coli*, to contain ~60 monomeric units [13].

The block of the channel by lanthanum is of particular interest. The Hill coefficient is very high, indicating strong

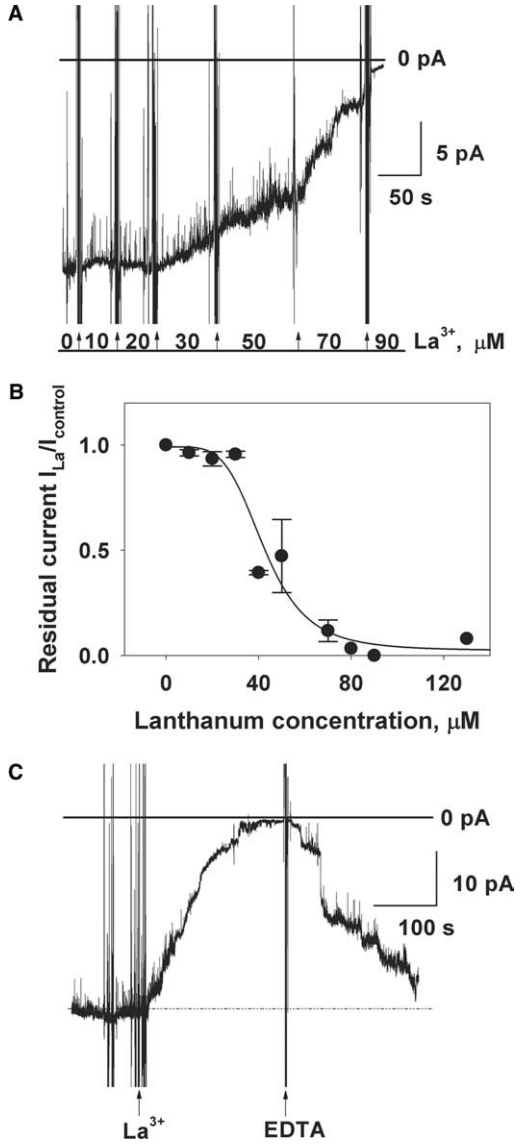


Fig. 3. Single-channel block induced by lanthanum. (A) Single channel recording in the presence of different concentrations of lanthanum. Recording solutions contained 150 mM KCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, and 5 mM Tris–HEPES, pH 7.4, holding potential −50 mV. (B) Concentration dependence of lanthanum block, $IC_{50} = 42 \pm 4$ μM, Hill coefficient (8.4 ± 1.2, from mean of fits to six individual experiments). (C) Recovery from lanthanum inhibition after EDTA addition. Recording solutions: 150 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM Tris–HEPES, pH 7.4. Arrows indicate addition of 70 μM $LaCl_3$ or 3 mM EDTA.

cooperativity, which probably results from the presence of multiple binding sites. Such steep concentration dependence is not the norm for protein channels which are blocked by lanthanum, but it is consonant with the model of PHB/$Ca^{2+}$/polyP complex organization, which proposes a large number of metal binding sites provided by polyP [1]. This striking lanthanum concentration dependence might provide an indication of likely PHB/$Ca^{2+}$/polyP dependent transport in in vivo experiments.

Although physiological roles of PHB/$Ca^{2+}$/polyP complexes remain to be established, it is known that their presence in bacterial membranes is required for bacterial competence [5]. One

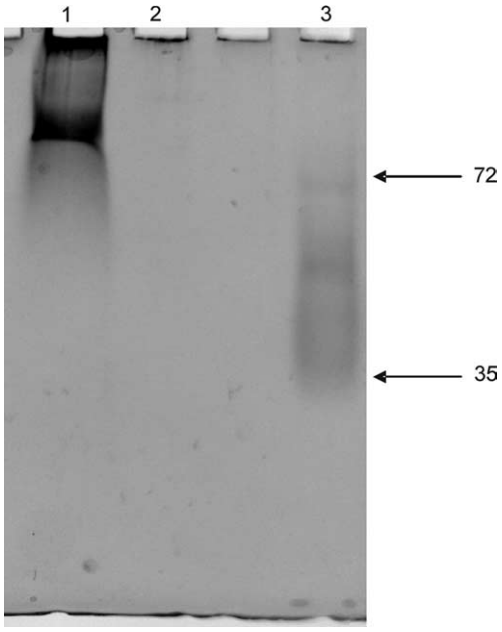


Fig. 4. Toluidine blue staining of polyP isolated from membrane fractions of competent (lane 1), or non-competent *E. coli* (lane 2), or from rat liver mitochondria (lane 3) in a 15% PAGE gel. The arrows indicate the position of the dye standards on the gel: 35 (no. of phosphate monomers) corresponds to the position of BPB, bromphenol blue; 72 to the position of XC, xylene cyanol. Amounts of polyP loaded on the gel were: for *E. coli*, the polyP extracted from 35 mL of *E. coli* at O.D. = 0.4, and for mitochondria, the polyP isolated from an aliquot of the mitochondrial fraction containing 30 mg protein.

hypothesis proposed that DNA import occurs through the PHB/$Ca^{2+}$/polyP complex by a mechanism in which DNA molecule replaces polyP inside PHB cylinder [1]. This mechanism was judged to be unlikely, based on the small physical size of PHB molecule in the model of the PHB/$Ca^{2+}$/polyP complex [14]. However, the conductances that we have observed suggest that the channel formed by the PHB/$Ca^{2+}$/polyP complex might permit transport of double stranded DNA [6]. Recently, it was shown that addition, to the medium, of synthetic PHB of the same size as in the PHB/$Ca^{2+}$/polyP complex of native bacterial cells, can induce DNA transformation in *E. coli* [15]. Thus, the possibility that PHB/$Ca^{2+}$/polyP is involved in DNA transport seems worthy of reexamination.

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